RNA helicase A's coordinate role with Tax and CBP/p300 in HTLV-1 biology

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by
Greeshma Allareddy

The Ohio State University
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Project Advisor: Professor Kathleen Boris-Lawrie, College of Veterinary Medicine

Abstract

Cancer is a multifaceted process and many of its hallmarks involve complex protein-protein interactions that dysregulate gene expression and involves yet-to-be understood activities of RNA helicase A (RHA), also known as DHX9. Human T-cell leukemia virus type 1 (HTLV-1) is a causative of adult T-cell leukemia/lymphoma (ATL), which has no cure. The viral oncoprotein, Tax, is necessary for cell transformation and transactivates gene transcription by associating with promoter DNA, various DNA binding proteins and histone acetyltransferases CBP/p300. Using co-immunoprecipitation assays (coIPs), we observed a physical interaction between CBP, RHA and RNA polymerase II. Likewise, coIPs demonstrated the physical interaction of RHA and HTLV-1's post-transcriptional control element (PCE), which is necessary for viral RNA translation. Thus we hypothesized that RHA is necessary for the transcriptional, as well as post-transcriptional expression of HTLV-1. RHA (DHX9) gene mutations and amplifications are cataloged in the Cancer Genome Atlas and their significance will be discussed. To test our hypothesis, HTLV-1 LTR-Luc reporter and *tax* expression plasmids were transfected into HEK293 and HeLa cells and Luc protein and luc RNA were quantified by immunoblot and RT-qPCR, respectively. Exogenous expression of FLAG-RHA (FLAG-RHA) increased HTLV-1 LTR-Luc activity, whereas downregulation of endogenous RHA by siRNA reduced Luc activity to basal levels. Exogenous expression of substitution mutants FLAG-RHA W339A and K417R reduced luc RNA and protein activity. We postulate the W339A mutation abrogated the necessary physical interaction between CBP, RHA and RNA polymerase II. CoIP experiments are needed to document our preliminary data indicating that RHA is necessary for CBP-dependent Tax-mediated HTLV-1 transcription, and its molecular basis is in tethering RNA polymerase II to CBP/p300 and Tax on the HTLV-1 promoter.

Contents

Introduction

RNA helicase A (RHA), an essential multifunctional protein

Cancer is a multifaceted process and many of its hallmarks involve complex protein-protein interactions that dysregulate gene expression. One protein we believe to be involved is host protein, RNA helicase A (RHA) [1]. RHA, also known as DHX9, is a highly conserved protein, homologous to bovine nuclear DNA helicase II and Maleless protein, Mle in *Drosophila melanogaster* [1] [2]. It is absolutely essential to the cell; RHA-knockout is embryonic lethal in mice [3] [4]. It is known to bind both single-stranded nuclei acids and unwinds double-stranded nucleic acids in an ATP-dependent manner [3] [5]. A predominantly nuclear protein, RHA regulates expression of both viruses and cells at the transcriptional and post-transcriptional levels [1]. It regulates cellular transcription by interacting with RNA Polymerase II (RNA Pol II) (by its minimal transactivation domain MTAD of RHA, aa 331-380) and bridging to DNA binding proteins and tethering transcriptional factors and coactivators such as breast cancer 1 (BRCA1) , nuclear factor-kappa B (NF-κB), and CREB-binding protein (CBP) [1] [2] [3] [5] [6] [7] [8].

RHA is necessary for promoting efficient translation of cellular proto-oncogenes and genes of the oncogenic retrovirus HTLV-1 [9] [10]. In HTLV-1, it does this by binding to an RNA element called the post-transcriptional control element (PCE), 160 nt stem-loop structure at the 5' leader of the mRNA (5' untranslated region). RHA performs similar functions in other retroviruses such as feline leukemia virus, spleen necrosis virus and human immunodeficiency virus (HIV) [10]. RHA has been notably associated with somatic neoplasms such as breast, prostate and lung [1]. Open questions revolve around the relationship between the transcriptional and post-transcriptional activities of RHA on oncogenes. Using HTLV-1 as a model, we

evaluated RHA's specific role in HTLV-1 gene expression using reporter gene analysis and co-immunoprecipitation.

Human T-cell lymphotropic virus type-1 (HTLV-1), an oncogenic retrovirus

Many viruses are responsible for infectious neoplasms in humans and animals. Oncogenic viruses cause approximately 12% of the known human cancers and among those is human T-cell lymphotrophic (leukemia) virus type 1 (HTLV-1) [11]. The existence of a human retrovirus was unknown to man until the late 1970's with the discovery HTLV-1. In the 1980's, HTLV-1's more well-known cousin HTLV-III, renamed today as human immunodeficiency virus (HIV-1) discovered [12]. In 2012, it was estimated that over 10 million people worldwide are infected with HTLV-1 [13]. Endemic to southwestern Japan, Sub-Saharan Africa, South America and the Caribbean, the infection is associated with HTLV-1 associated myelopathy/tropical spastic paraparesis (HAM/TSP), and our focus, the highly aggressive adult T-cell leukemia/lymphoma (ATL) [14]. Approximately 3-5% of those infected progress to adult T-cell leukemia for which there is no treatment [15].

HTLV-1 falls under viral family *Retroviridae* along with HIV-1, feline leukemia virus, equine infectious anemia virus, etc. Its virion structure, genetic make-up and entry receptor are known [15]. Thirty-six years after its discovery, the exact mechanisms of oncogenesis are still being discovered. It is known however that HTLV-1 encodes the oncoprotein called Tax, and several cellular proteins such as NF-κB and CBP, which are binding partners of RHA, have been identified to participate with Tax to activate gene expression [8] [7] [16]. Thus we sought to elucidate the coordinate activity of Tax, CBP, and RHA.

Tax, a viral oncoprotein critical for HTLV-1 promoter activity

HTLV-1 encodes the viral oncoprotein Tax. Tax is crucial to viral replication and pathogenesis in both early and late stages. In the nucleus, Tax is absolutely required for

transcription from the viral promoter; it is also affects transcription of cellular genes. In the cytoplasm, Tax can activate the NF-κB pathway and indirectly regulate multiple genes and pathways that control cellular growth such as those that control proliferation and apoptosis. Tax's deregulation of these important pathways causes cellular transformation [16] [17]. A minority of patients progresses to ATL, but in those patients, 50% have Tax expression silenced, signifying that Tax is important in instigating neoplasms, but not sustaining them [18].

As a transcriptional activator, Tax promotes efficient viral gene expression by binding promoter DNA and DNA-binding proteins that recruit basal transcriptional machinery. Specifically, Tax associates with a set of three 21 bp repeat sequences in the viral promoter region in the unique 3' (U3) region of the long terminal repeat (5' LTR), in combination with phosphorylated cAMP response element binding (pCREB) protein, another cellular transcription factor. These sequences, called the Tax-response elements (TREs), are similar to the cellular cAMP-response elements (CREs) to which CREB binds [14] [19]. Tax does not physically bind to TREs, which are bound by CREB, but associates with GC-rich sequences flanking the TREs.

Tax mediated activation of cellular CRE-containing promoters is dependent on the presence of phosphorylated CREB (pCREB) [19] [20]. Protein kinase A (PKA) is the cAMP-activated kinase that is largely responsible for phosphorylating CREB at Ser-133 [21]. A phosphorylated CREB then functions coordinately with Tax to recruit other transcriptional coactivators such as CBP/p300.

CBP/p300, players in transcription and cellular transformation

Cellular proteins tying together the two ends of our story are DNA coactivators: CREB-binding protein (CBP) and paralog E1A-binding protein (p300). Though each protein does have its own distinct role in certain biological settings, CBP and p300 are generally held to be

functionally redundant [22]. CBP/p300 is a transcriptional coactivator that functions with basal transcription factors and other DNA binding proteins to activate transcription of a multitude of genes. Specifically CPB/p300 has histone acetyltransferase (HAT) activity that regulates chromatin. In cells chromatin is organized into nucleosomes, complexes of histone proteins with DNA tightly wrapped around them, so that most of the DNA is inaccessible to basal transcriptional factors and other transcriptional machinery. CBP/p300's HAT activity modifies histones via acetylation, which causes the nucleosome to be more accessible to transcriptional machinery. Their HAT domains are also capable of modifying other transcription factors to regulate their activity [22] [23].

CBP/p300 frequently interacts with important transcriptional coactivators of RNA Pol II-dependent transcription, including basal transcription factors such as TATA-binding protein that are necessary to initiate transcription [22]. RHA is an important mediator that functions by bridging RNA Pol II and CBP/P300 transcriptional coactivation complex. Specifically, the N-terminal domain of RHA (aa 1-250) binds to the C/H3 region (aa 1805-1890) of CBP [8]. Both cellular and viral genes are transcriptionally responsive to CBP/p300 tethered by Tax to other DNA-binding proteins at the viral TREs. CBP's KID interaction (KIX) domain (aa 588 - 683) simultaneously binds to **phosphorylated** CREB's kinase interaction domain (KID; aa 100-160) and Tax's KID-like domain (aa 81-95). CBP also interacts with Tax at a second domain, the C-terminal transcriptional activating domain (CR2; aa 312-319), indicating that Tax/CBP interaction is important at the viral promoter [24].

CBP/p300 has an integral role in cancer. Chromosomal translocation involving CBP/p300 genes have been connected with leukemia/lymphoma. CBP/p300 not only functions with tumor-suppressor proteins such as p53, but also with oncogenic transcriptional co-activators

such as c-*jun*. Both CBP and p300 are targets for DNA viruses that cause cellular transformation, such as Epstein-Barr virus (EBV) [23] [25].

Considering all these observations, we have investigated the molecular basis for the coordinate interaction of RHA, Tax and CBP/p300 in the regulation of viral gene expression, which ultimately leads to cellular transformation. Our experimental hypothesis is **that RHA is necessary for Tax–mediated, CBP-dependent viral gene transcription (Fig. 1).**

Significance of this study

Protein-protein and DNA-protein interactions significantly contribute to mechanisms of disease and viruses utilize these interactions in their replication. Studying the cooperative effects of RHA, CBP/p300 and Tax on gene transcription is a necessary step to uncover RHA/viral protein and host protein/viral protein interactions in HTLV-1 and other viruses and to expose their instruments of infection.

In cancer, the web of such interactions is typically altered from normal cell biology in a myriad of ways. Looking at somatic mutations in disease and altered interactions provides a key to understanding at the molecular level and knowledge on how cell biology can be changed or reverted back to its original state. Understanding the interface between RHA, CBP/p300 and Tax is important to dissecting ATL caused by HTLV-1 and to other cancers in somatic neoplasms. RHA's gene alterations in different cancers through bioinformatics from the Cancer Genome Atlas (TCGA) are identified and provide insight on specific copy number changes or mutations of the gene [26] [27]. These alterations may well be the same ones used by oncogenic viruses for their own use and to facilitate cellular transformation.

To this day there is a dearth of knowledge on how ATL arises in patients infected with HTLV-1 and in long term, this information is necessary to therapeutically halt the virus and

eventually the cancer. The knowledge obtained from this study can be tied back to cancer studies in general. The viral model of RHA, CBP/p300 and Tax interactions may exist in cancerous cells in the form of other CBP/p300 complexes, functioning with other DNA binding adaptor proteins like Tax, that require RHA for the transcription of specific genes. Such cellular transcription models are especially important in the context of cancer and gene regulation during cellular transformation. In conclusion, this study has a lot to contribute to two very important fields of retrovirology and oncology.

Methods

Plasmids

The previously described plasmids used in this study are empty-FLAG plasmid (pCDNA) and FLAG-RHA plasmid [28]. K417R and W339A plasmids were made based on previous literature [3] [8]. pRenilla was obtained from Promega.

CMV-Tax expression plasmid, CMV-HBZ expression plasmid & HTLV-1 LTR-luc reporter gene plasmid were obtained from Dr. Patrick Green (The Ohio State University). GFP-Tax and wild-type PKA (WT-PKA) plasmids were obtained from Dr. Susan Marriott (Baylor College of Medicine).

Cells and transfections

HEK293 cells were grown in EMEM (ATCC) with 10% fetal bovine serum and 1% antibiotic/antimycotic. For the overexpression studies, HEK293 cells (5×10^5 cells/plate in 6-well plates) were transfected with pCDNA (1 µg), wild-type FLAG-RHA (1 µg) and RHA mutant (2 µg each) after 24 hrs, followed by Tax (0.2 µg) plasmid transfection and HTLV-1 LTR-Luc (0.1 µg) plasmid cotransfection after 48 hrs with X-tremeGENE HP (Roche) at a 1:3 DNA to X-tremeGENE ratio, according to the manufacturer's protocol.

HeLa cells were grown in EMEM (ATCC) with 10% fetal bovine serum and 1% antibiotic/antimyotic. For the downregulation studies, HeLa cells (2×10^5 cells/plate in 6-well plates) were reverse transfected with siRNA complementary to the RHA mRNA or nonsilencing control siRNA consisting of a scrambled (Sc) sequence at a final concentration of 10 nM in Lipofectamine 2000 (Invitrogen), Opti-MEM media (Invitrogen) and EMEM (ATCC) with 10% fetal bovine serum, according to a published protocol [29]. Cells were rinsed twice and freshly

incubated in EMEM (ATCC) with 10% fetal bovine serum and 1% antibiotic/antimyotic for 24 hrs. Cells were then cotransfected with CMV-Tax (0.4 µg) and CMV-HBZ (0.4 µg). At 48 hrs, cells were cotransfected with HTLV-1 LTR-Luciferase (0.1 µg) and pRenilla (0.025µg). All transfections were carried out with X-tremeGENE HP (Roche) at a 1:3 DNA to X-tremeGENE ratio, according to the manufacturer's protocol.

Luciferase assay and RNA analysis

HEK293 and HeLa cells were scraped, centrifuged and lysed with 1X Passive Lysis Buffer (Promega) following the manufacturer's protocol. Cellular lysates were then treated with Luciferase Assay Reagent (Promega) following the manufacturer's protocol and photoluminescence measured by a standard luminometer. For cells transfected with pRenilla, additional reagents were added and the Dual Luciferase (Promega) protocol was followed.

Total cellular lysates obtained for the Luciferase assays were also used for RNA extractions. Total RNA was isolated with Trizol LS reagent (Invitrogen) and purified with RNAeasy MinElute Cleanup Kit (Qiagen) following the manufacturer's protocol. RNA concentration was determined by spectrophotometer (NanoDrop). Isolated RNA was treated with Turbo DNA-free Kit (Life Technologies) and used to prepare cDNA in a 20 uL-reaction mixture with Omniscript reverse transcriptase (Qiagen) following the manufacturer's protocol. Quantitative PCR was performed with 2 uL of cDNA with CFX96 Real Time System (BioRad). Primers KB1747 Luc and KB1748 Luc were used to amplify the PCR product. RNA samples without reverse transcriptase were used as negative controls. Details on primer sequences are available on request.

Immunoprecipitation and immunoblot analysis

HEK293 and HeLa cells were grown in EMEM (ATCC) with 10% fetal bovine serum and 1% antibiotic/antimyotic. HEK293 cells (1.5×10^6 cells/plate in 10 cm plates) were serially transfected with FLAG-RHA (2 µg) and GFP-Tax (2µg) 24 hrs and 48 hrs after plating, respectively. Cells were scraped, washed and lysed in RIPA buffer (50 mM Tris pH 8.0, 0.1% SDS, 1% Triton-X, 150 mM NaCl, 1% deoxycholic acid, 1% protease inhibitor cocktail, 0.1% DTT). Sepharose pre-conjugated FLAG beads (Sigma Aldrich) were blocked with 0.5% BSA and washed with NETN-150 buffer (0.5% NP40, 0.1 mM EDTA, 20 mM Tris-HCl pH 7.4, 150 mM NaCl). Lysates were added to the beads and incubated for 3 hrs at 4°C. Following the incubation, complexes were washed thrice with NETN-150 buffer and twice with wash buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl). IP efficiency was assessed by immunoblotting equivalent aliquots of input and flow-through lysates. A control IgG rabbit IP was performed alongside.

HeLa cells (1×10^6 cells/plate in 10 cm plates) were transfected with WT-PKA (2.5 µg), scraped, washed and lysed in RIPA buffer. The lysates with and without PKA (control) were added to IgG mouse and IgG rabbit (Sigma Aldrich) conjugated Protein G magnetic beads (Invitrogen) for 30 min at 4°C in order to pre-clear the lysates. Pre-cleared lysates were then added to RHA (Vaxron) conjugated Protein G magnetic beads (Invitrogen) and incubated for 2 hrs at 4°C. Following the incubation, complexes were washed thrice with NETN-150 buffer. IP efficiency was assessed by immunoblotting equivalent aliquots of input and flow-through lysates.

SDS-PAGE was performed by loading equal amounts of protein (5 or 10 µg) or equal loading volumes of 40 uL (only for the co-IP assays) on 4-15% Mini-Protean TGX™ gradient

gels (Bio-Rad). Proteins were transferred onto nitrocellulose membranes and probed with appropriate antibodies. The antibodies used for immunoblotting were: FLAG (1:5000, Sigma-Aldrich F7425), α-tubulin (1:1000, Santa Cruz sc-23948), RHA (1:7500, Vaxron PA-001), CBP (1:500, Santa Cruz sc-7300) and Pol II (1:200, Santa Cruz sc-9001).

Statistical analyses

The Luciferase assays were conducted in triplicate with three independent samples for each condition. RT-qPCR was also conducted in technical triplicate with three reactions from one sample of each condition. CoIPs were conducted with individual samples without replicates. Two-tailed student t- tests were used to test significance comparing *p-values* with $\alpha = 0.05$.

Bioinformatics of the Cancer Genome Atlas (TCGA)

The results discussed below are in whole or part based upon data generated by the Cancer Genome Atlas (TCGA) Research Network: http://cancergenome.nih.gov/. cBioPortal for Cancer Genomics was primarily used for simplifying data analyses of the data provided by TCGA and generating the results below. TCGA contains both published and unpublished data sets (indicated as provisional data). The cBioPortal site only stores detailed mutation data for published cancer studies as the National Cancer Institute guidelines prevent provisional data from being redistributed until they have been validated [26] [27].

Results

RHA downregulation negatively affects Tax-dependent HTLV-1 gene expression

To examine the effect of RHA downregulation on Tax-mediated CBP-dependent activity of the HTLV-1 promoter, luciferase assays were conducted with HTLV-1 LTR-Luc reporter plasmid, Renilla expression plasmid for transfection efficiency control, and Tax expression plasmid with and without siRNA mediated down regulation of RHA. The HTLV-1 LTR-Luc reporter plasmid contains the 3' LTR viral promoter upstream of the Luciferase gene (Fig. 3). siRNAs were transfected initially followed by Tax transfection and lastly HTLV-1 LTR-Luc plasmid transfection into HeLa cells. Successful knockdown (KD) was confirmed by WB analysis (Fig. 4). Firefly luciferase (Luc) activity was measured and normalized to Renilla luciferase expression. In the absence of Tax, siRNA-mediated RHA KD did not have any effect on Luc activity. In the presence of Tax, however, siRNA-mediated RHA KD resulted in a decrease of Luc activity by a factor of 20 compared to Luc activity of cells without RHA KD. Noticeably, these levels were approximately equal to Luc with RHA KD but without Tax expression, indicating that both RHA and Tax are required at threshold levels for HTLV-1 gene expression (Fig. 4).

RHA overexpression positively affects Tax-dependent transactivation of the HTLV-1 promoter

After observing that minimal levels of RHA are required for HTLV-1 promoter activity, we decided to examine the effect of RHA overexpression on Tax-mediated CBP-dependent activity of the HTLV-1 promoter. Luciferase assays were conducted with HTLV-1 LTR-Luc reporter plasmid, Tax, pCDNA, FLAG-RHA, K417R and W339A expression plasmids. pCDNA is an empty-FLAG control plasmid. K417R is a mutant RHA plasmid containing a Lys to Arg

point mutation at aa 417 in the ATP-binding domain of RHA that is necessary for RHA's helicase activity and important both for transcription and translation [8] [28]. W339A is a mutant RHA plasmid containing a Trp to Ala point mutation at aa 339 in the minimal transactivation domain (MTAD) of RHA that interacts with RNA Polymerase II (RNA Pol II) and is necessary for CREB-dependent transcriptional activation [3]. We expected luciferase activity to decrease under the mutant transfection conditions. The RHA expression plasmids were transfected 24 hrs after the cells were plated, followed by co-transfection of Tax and HTLV-1 LTR-Luc plasmids at 48 hrs. Transfection of the RHA expression plasmids was confirmed by Western blot (WB) analysis. Firefly luc activity was measured and statistically analyzed.

In the presence of Tax, cells overexpressing wild-type RHA (FLAG-RHA) presented a 5-fold increase of Luc activity compared to the pCDNA control. Cells transfected with RHA mutants K417R and W339 presented a decrease in Luc activity by a factor of 8 and a factor of 15, respectively relative to the Luc activity of FLAG-RHA overexpressing cells (Fig. 5). Without Tax, luciferase expression is too low even with wild-type RHA overexpression (figure not shown). These observations show that Tax-dependent transcriptional activation of the HTLV-1 promoter increases in response to RHA overexpression, but decreases when RHA mutants are overexpressed. The results indicate that K417R and W339A mutants have a dominant negative effect and that the MTAD and ATP-binding domains of RHA are essential for RHA and Tax's collective functionality in regulating HTLV-1 gene expression.

To discern between a transcriptional and post-transcriptional level of interaction, we conducted quantitative real-time reverse transcriptase polymerase chain reactions (RT-qPCR). Total RNA was extracted, DNAse treated and converted to cDNA, followed by primer-specific

amplification of luciferase messenger RNA (luc mRNA). The mRNA was quantified based on a standard curve and normalized by volume. The results showed significant decreases of luc mRNA in response to mutant K417R and W339A compared to the control pCDNA condition by a factor of 1.5 and a factor of 4, respectively (Fig. 6). The luc mRNA levels showed significant decreases in response to mutant K417R and W339A compared to the FLAG-RHA condition by a factor of 7 and a factor of 19, respectively. The luc mRNA levels showed a 4-fold increase when FLAG-RHA was overexpressed compared to the luc mRNA levels of the pCDNA control.

Further analysis through the comparison of Luciferase activity/luc RNA ratios demonstrated no significant difference between basal RHA expression (pCDNA), and wild-type overexpression (FLAG-RHA) and overexpression of K417R mutant (Table 1). This shows that FLAG-RHA solely altered the transcriptional efficiency of luc RNA but not the translational efficiency. The K417R mutant, being important for both transcription and translation decreased both luc RNA levels and protein significantly compared to pCDNA condition so it is expected that this condition's Luc activity/luc RNA ratio was not significantly different from the pCDNA condition. Unexpectedly, the W339A mutant expression, despite decreasing both luc RNA levels and protein significantly compared to pCDNA condition, significantly increased the Luc activity per RNA copy implying RHA's role in increasing translational efficiency. The increase in translation efficiency was able to compensate for decrease transcriptional efficiency to produce more Luc activity per copy of luc RNA. This result in particular suggests a dual role of RHA at both transcriptional and translational levels (Fig. 1).

Further experiments need to be conducted with the mutants to discern exactly how much of a role RHA plays at both transcriptional and post-transcriptional stages and in what settings. Despite the lack of significant differences between the Luc activity/luc RNA ratios when

comparing the control (pCDNA) to FLAG-RHA and K417R mutant conditions, the significant increases and decreases in Luc protein and luc RNA alone suggest that either transcriptional or translational efficiency is being increased (Table 1). For example, K417R mutant might cause a decrease in transcriptional efficiency as shown by the significant decrease in the mRNA number compared to the control, but perhaps it is still able to function to enhance translational efficiency or the endogenous RHA could be sequestered to the cytoplasm to function post-transcriptionally, which is why we haven't observed a significant decrease in the Luc activity/luc RNA ratio compared to the control. Experiments tailored to discern RHA's function only at the transcriptional or post-transcriptional level in this context can illuminate the protein's specific roles.

RHA and Tax are capable of binding to each other in a biological setting

Based on the coordinate functional activity between RHA and Tax as suggested by the downregulation and overexpression studies, we decided to test whether RHA and Tax' functional role had a physical, molecular basis. Co-immunoprecipitation (CoIP) assays were performed to see if the proteins were part of the same transcriptional complex. After plating, HEK293 cells were transfected with FLAG-RHA (2 µg) at 24 hrs and GFP-Tax (2µg) at 48 hrs. Soluble total cell lysates were immunoprecipitated with pre-conjugated anti-FLAG beads. Western blot analysis of the protein complexes in the FLAG immunoprecipitant was performed using an anti-GFP antibody and it confirmed that GFP-Tax was co-immunoprecipitated (Fig. 7). A positive control, CBP80 and a negative control, eIF4E were also analysed via WB to validate a successful CoIP. Our lab has shown that CBP80 does bind to RHA whereas eIF4E does not (unpublished data, Boris-Lawrie, K.). Both low and high exposures were conducted. WB analysis suggested that Tax has an affinity for RHA and they are capable of binding to each other *in vivo*.

To validate the previously published interactions of RHA with CBP and RNA Pol II, endogenous CoIP assays were performed with HeLa cells. HeLa cells were transfected with WT-PKA (2.5 µg) 24 hrs after plating. Cells were scraped and lysed. Lysates were added to magnetic beads conjugated with IgG mouse antibody for pre-clearing. Pre-cleared lysates were immunoprecipitated with RHA antibody-conjugated magnetic beads. Immunoblot analysis of the protein complexes in the RHA immunoprecipitate confirmed that CBP and RNA Pol II were co-immunoprecipitated (Fig. 8).

Genotypes of RHA (DHX9) in tumors, bioinformatics of patient neoplasms

The Cancer Genome Atlas (TCGA) is a large project that aims to catalogue all the mutations involved in different cancers through the use of sequencing and bioinformatics. cBioPortal for Cancer Genomics is an online resource that provides user-friendly ways to analyze and manipulate the genomic data (published and unpublished) provided by TCGA [26] [27]. One of these ways is to access the data sets based on a single gene, in our case, *RHA* (listed as *DHX9* in the database). Ways to view the data sets include alteration frequency bar graphs, mutation plots over the protein domains, and multi-categorical tables.

To examine how frequently *RHA* was altered in various cancers, alteration frequency bar graphs were generated looking at how frequently the gene was "totally altered" (including all changes and mutations), amplified and mutated. Five specific studies (selected based on having the highest "total alteration" frequencies) and two studies on acute myeloid leukemia (AML) were selected (Fig. 9A). Those five studies were also highlighted on frequency bar graphs representing copy number alterations (CNA) and mutations exclusively (Fig. 9B,C). This information is also organized in table format (Table 2). Two breast cancer studies (breast cancer patients xenografts and breast invasive carcinoma), one liver cancer study (hepatocellular

carcinoma), one pancreatic cancer study (pancreatic adenocarcinoma) and one uterine cancer study (uterine carcinosarcoma) were among the selected studies for having the highest total alteration frequencies of *RHA.*

A horizontal plot with all of RHA's mutations and their frequency of occurrence among all catalogued cancer studies was generated (Fig. 9D). Specifically, mutations of three select regions are highlighted on the plot. These regions of interest include RHA's N-terminal domain (aa 1-250) that interacts with CBP, minimal transactivation domain (MTAD at aa 331-380) that interacts with RNA Polymerase II and a region overlapping the double-stranded RNA-binding domain 2 (dsRBD2, aa 230-325) that interacts with BRCA1 (Fig. 9E). Mutations in these three regions that were observed in the any of the selected seven cancer studies and their predicted functional impact score are shown in a table (Table 3). Specifically, four missense mutations D206G, I23V, V40G, and L117R were found in studies for liver, hepatocellular carcinoma, breast invasive carcinoma, pancreatic adenocarcinoma and uterine carcinosarcoma, respectively. All of these mutations are found in RHA's N-terminal domain in a region that bridges CBP to RNA Polymerase II. Further studies on the mutations' functional impact will reveal the necessity of RHA/CBP interaction for proper gene expression regulation, which, when disrupted, can lead to cellular transformation.

An alteration frequency bar graph highlights five studies among all cancer studies based on highest amplification or deletion type alteration frequencies (Fig. 10). These studies include one breast cancer study (breast invasive carcinoma), one liver study (hepatocellular carcinoma), one lung study (lung adenocarcinoma), one uterine cancer study (uterine carcinosarcoma) and one ovarian cancer study (ovarian serous cystadenocarcinoma). An alteration frequency bar graph also highlights five studies among all cancer studies based on highest mutation type

alteration frequencies (Fig. 11). These studies include two bladder cancer studies (bladder urothelial carcinoma, published and unpublished), one pancreatic cancer study (pancreatic adenocarcinoma), one colorectal cancer study (colorectal adenocarcinoma) and one lung cancer study (small cell lung cancer). These results indicate the prevalence of RHA gene alterations in a wide range of cancers.

Lastly, all "validated" mutations of *DHX9* are shown in a multi-categorical table generated by cBioPortal (Fig. 12). Most of these mutations pertain to colorectal cancer. In particular, three colorectal cancer studies show valid missense mutations in our regions of interest. Ovarian and lung cancer studies also show valid mutations. In particular, the lung cancer study shows a missense mutation in RHA's MTAD region. This table is useful way to explore a particular sample and/or cancer study. It also allows users to view the information through different categories by viewing all mutations in numerical order based on amino acid, assessing highest functional impact mutations, examining the validation status and more.

Role of RHA's amplification at transcriptional and post-transcriptional levels

The overexpression of wild-type RHA (FLAG-RHA) resulted in significant increases of luc mRNA transcription and Luc protein activity. In the context of the bioinformatics results described above, it can be implied that similar effects are being observed on RHA-mediated gene expression in those cancer studies that show copy number amplification of RHA (DHX9). Through gene amplification, increased RHA expression can subsequently upregulate transcription and translation of proto-oncogenes in cancerous cells. These implications open up new possibilities for future experimental directions as well as the mechanisms underlying cellular transformation.

Discussion

RHA as a transcriptional regulator

RNA helicase A (RHA) is a multifunctional host protein that has been shown to regulate gene expression at both transcriptional and translational levels. Its cellular transcriptional interactions with p300/CBP histone acetyltransferase proteins and RNA Polymerase II implicated a potential coordinate role with Tax, a viral oncoprotein that interacts with p300/CBP and other DNA binding elements in the viral context of HTLV-1 infection. Here, we demonstrate the functionality of that partnership by showing the effects of RHA overexpression and downregulation with and without Tax. Indeed, the overexpression of RHA protein in conjunction with basal levels of Tax upregulates the expression of a reporter gene that contains the HTLV-1 viral promoter. On the contrary, siRNA-mediated downregulation of RHA despite having basal levels of Tax significantly lowers the expression of the same reporter gene. The functional coordination between the two proteins is necessary to have an effect on gene transcription as shown by the insignificant changes in reporter gene transfection under RHA overexpression or downregulation when Tax is absent, indicating that Tax is a necessary player.

The decreased reporter gene expression during the overexpression of RHA mutants K417R and W339A is an important indication of two things: the mutants are functioning as previously reported (dominant negative in the cellular context) and that not only do they have a similar role in the specific viral context of HTLV-1 infection but also that RHA is actively regulating gene expression at a transcriptional level.

Quantitative analysis of the reporter gene mRNA revealed further insight of the nature of the functional interaction between RHA and Tax. Decreased mRNA levels of the reporter gene during the overexpression of the RHA mutants affirm previous literature regarding the transcriptional importance of the specific amino acids belonging to the select domains of RHA,

in addition to indicating similar transcriptional roles and importance of said amino acids in the context of viral gene expression.

RHA as a post-transcriptional regulator

Further analysis through the comparison of Luciferase activity/luc RNA ratios presented expected and unexpected results. There was no significant increase in the ratio when comparing FLAG-RHA and K417R mutant condition to the pCDNA control but there was a significant increase in the ratio for the W339A mutant condition. Wild-type RHA (FLAG-RHA) functioned to increase transcriptional efficiency of Luc reporter gene with normal translational efficiency as indicated by the increase in both Luc activity and luc mRNA. The K417R mutant condition maintained a statistically similar Luc activity/luc RNA ratio by decreasing efficiency of transcription and maintaining normal translational activity as indicated by the decrease in both Luc activity and luc mRNA.

The W339A mutant condition unexpectedly resulted in a significant increase in Luc activity/luc RNA ratio. Though both Luc activity and luc mRNA levels significantly decreased in the W339A condition, the fact that the Luc activity/luc RNA ratio increased indicates increased translational efficiency. Since this mutant significantly affects RHA's transcriptional activity by preventing its proper interaction with RNA Pol II, it is possible that RHA is being recruited to a post-transcriptional complex to increase translational efficiency in order to compensate for the decreased transcriptional efficiency. This not only implies that RHA plays a dual role in viral gene expression but also that context plays an important role in directing RHA's degree of involvement at the transcriptional and post-transcriptional levels.

RHA tethers Tax to RNA Pol II

The interaction between RHA, CBP and RNA Pol II and potential link between RHA and Tax, further supports our hypothesis of RHA being a key host-factor in CBP/p300-dependent gene regulation of the viral genome. While a lack of proper antibody for Tax makes it difficult to assess endogenous/untagged Tax and RHA interaction, successful pull-down of GFP-Tax with RHA IP is indicative of the affinity between these two proteins. On the other hand, seeing that CBP and RNA Pol II were not pulled down when GFP-Tax was transfected into cells (figure not shown) indicates a possible post-transcriptional interaction between RHA and Tax, away from transcription factors such as CBP and RNA Pol II. Combining the new data regarding RHA and Tax's potential interaction and RHA's already established transcriptional bridging role, it is likely that RHA functions to tether Tax to RNA Pol II for efficient transcription of the viral genome.

In conclusion, much of data supports our hypothesis that RHA is necessary for CBP-dependent viral gene regulation at the transcriptional level, most likely by linking Tax to RNA Pol II, in addition to supporting a dual role played by RHA in conjunction with Tax at the post-transcriptional level.

RHA alterations are prevalent in selected cancers

Literature supports RHA's importance in cancers such as breast, prostate and lung, but using a bioinformatics approach to survey RHA's prevalence in other cancers has shown that it is altered in many different cancers. cBioportal analysis of TCGA reveal that RHA is altered in a total of 55 different types of cancer studies. Some studies show only RHA copy number variation such as the BCCRC Breast Xenograft studies, some studies only show RHA mutations such as Genentech's Colorectal Cancer studies and a majority of studies show that RHA is both

altered in number (copy number variation; CNA) and gene structure (mutations). The occurrence of RHA alterations in such a wide variety of cancers suggests many possibilities. RHA might have a common function or might be a part of a common pathway that is altered or deregulated during cellular transformation. Since so many studies show RHA amplification, there is also the possibility that normal RHA is just playing an abnormal role in the cell.

As TCGA contained no data on ATL, we hoped to examine data on another blood-borne cancer: Acute Myeloid Leukemia. Data analysis showed that only 0.5% of samples in two groups of AML studies showed RHA CNA and no data were found to indicate any RHA mutations in AML. These results are inconclusive without further studies about RHA's involvement in ATL.

We explored and analyzed RHA's mutation data based on the possibility that RHA's normal function or role in cellular pathways being altered leads to cellular transformation. Based on our hypothesis, that **RHA is necessary for Tax–mediated, CBP-dependent viral gene transcription** and that these interactions might be of importance in cellular transformation, mutants in relevant domains of RHA were explored. Four missense mutations D206G, I23V, V40G, and L117R were found in RHA's N-terminal domain in a region that bridges CBP to RNA Polymerase II. There is a possibility that each of these mutations, by disrupting the RHA/CBP interface and therefore preventing RNA Pol II to associate with CBP, are contributing to dysregulation of gene expression that might contribute to cellular transformation in the long-term.

Future Directions

The results of this study support our hypothesis that **RHA is necessary for Tax-mediated, CBP-dependent viral gene transcription.** While further studies can be conducted in any of the areas in this study, two possible future directions involve further examining RHA's role in the context of HTLV-1 biology and/or the context of cancer biology.

To elucidate RHA's exact mechanisms and role in HTLV-1 infection and transformation, the virus-host interface can be studied endogenously; experiments similar to ones conducted in this study can be performed in HTLV-1 infected T-cells (SLB-1 cell line). Protein-protein interactions involving RHA, Tax, CBP/p300 and other relevant proteins can also be studied using patient tissue samples to place molecular discoveries in the direct context of HTLV-1 disease.

The second area of exploration involves discovering and understanding RHA's role in cancer biology. Further bioinformatics exploration might reveal significant mutations of RHA which can then be cross-checked against mutations of RHA-binding proteins such as CBP/p300 in other cancers. That information can be used to understand RHA and its binding partners' role in instigating or sustaining neoplasms, for example through cell transformation assays using mutant forms of the proteins.

Lastly, long-term studies can be directed towards hindering RHA's interaction with certain proteins in certain pathways that contribute to cellular transformation or tumor sustenance or supporting an opposite role of RHA through drug development. Several drugs in the market already target CBP/p300 at specific sites and new drugs that target RHA's specific domains to hinder or promote its activity can be developed [23].

Figures

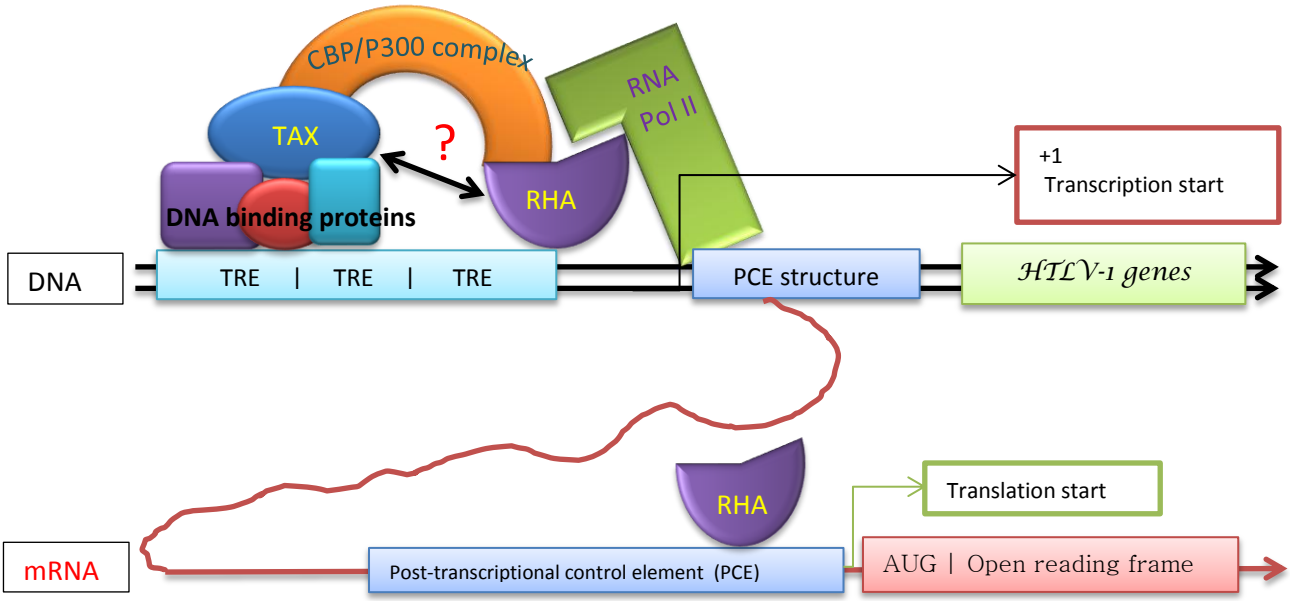


Figure 1. Our current working model of RHA in HTLV-1 transcription and translations

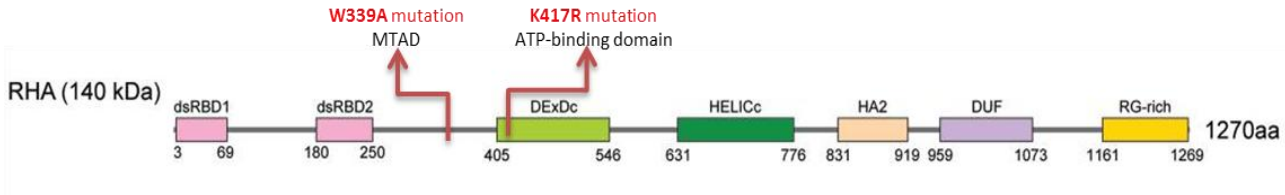


Figure 2. RHA's domain structure and mutation locations. Adapted from [2]).

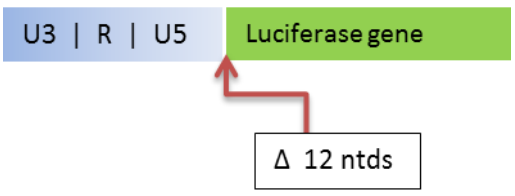


Figure 3. HTLV-1 LTR-Luc reporter plasmid with the 5' LTR viral promoter and Luciferase gene.

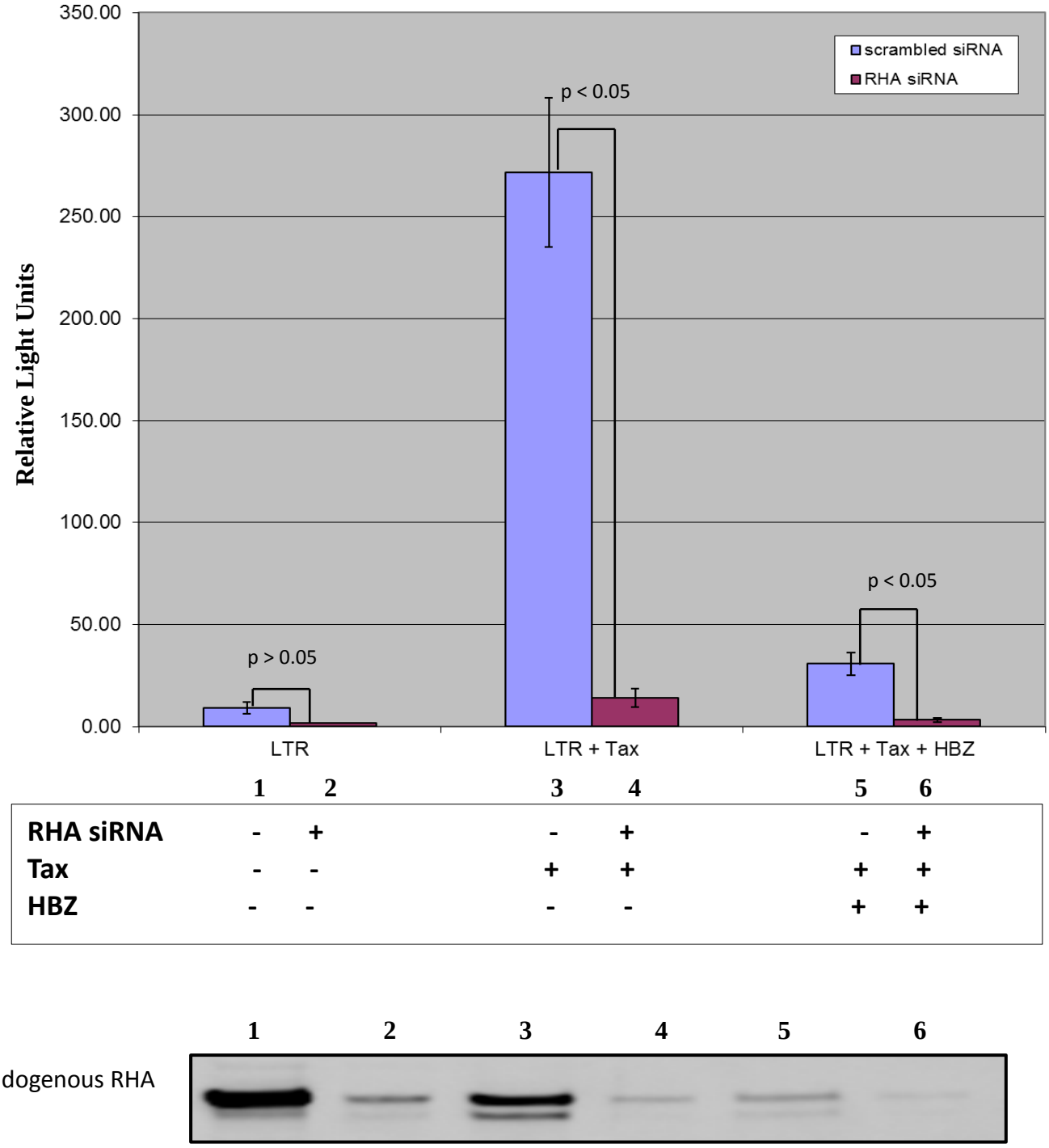


Figure 4.
siRNA-mediated downregulation of RHA significantly decreases Luciferase protein activity in the presence of Tax.

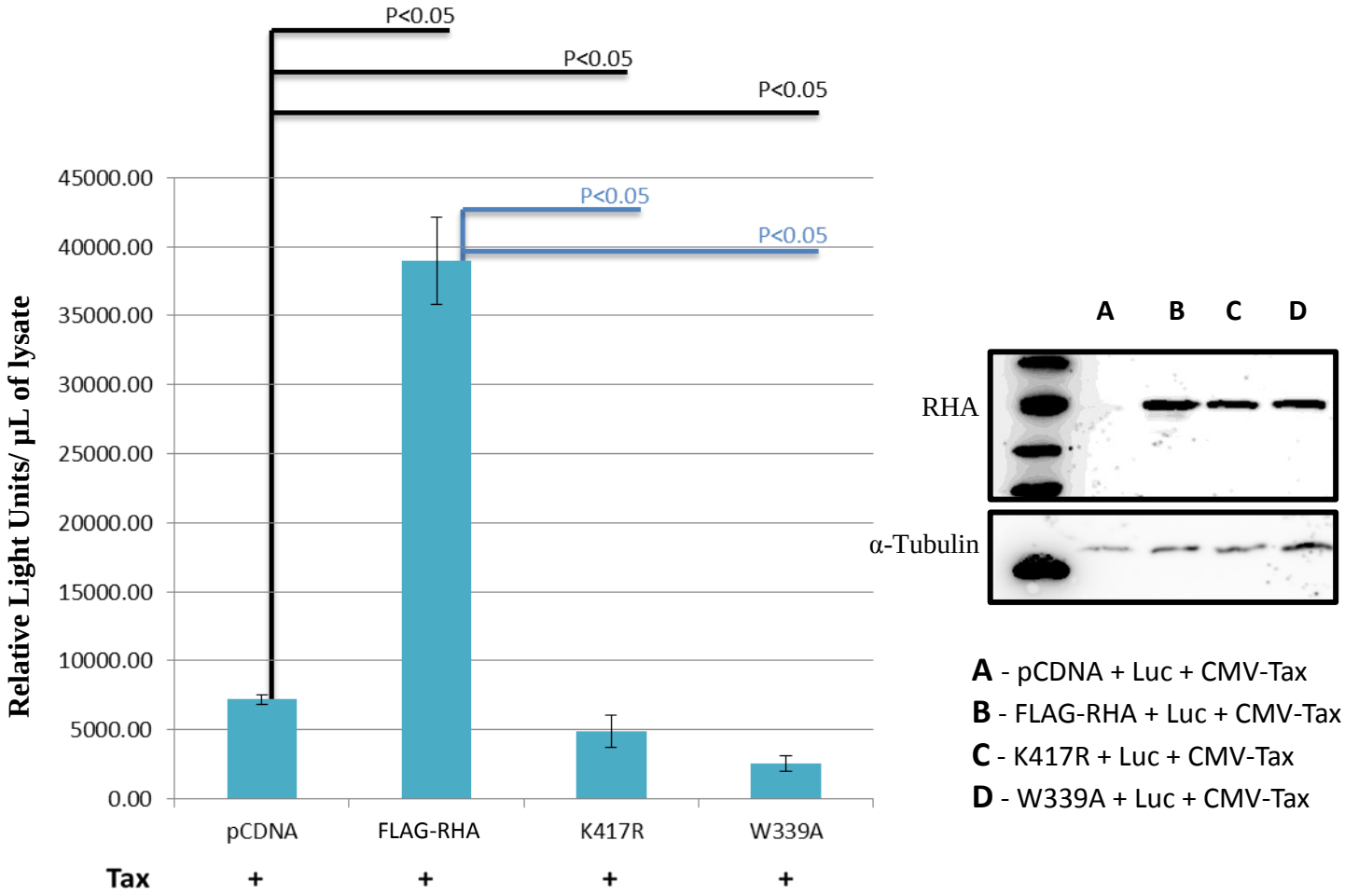
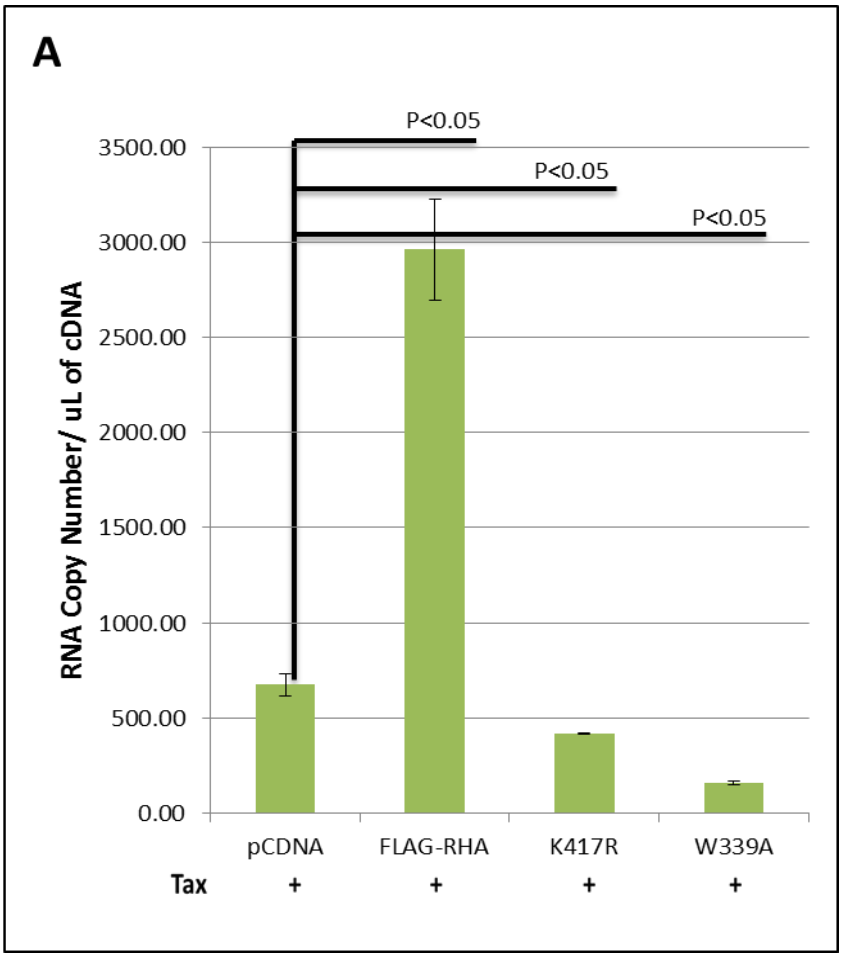
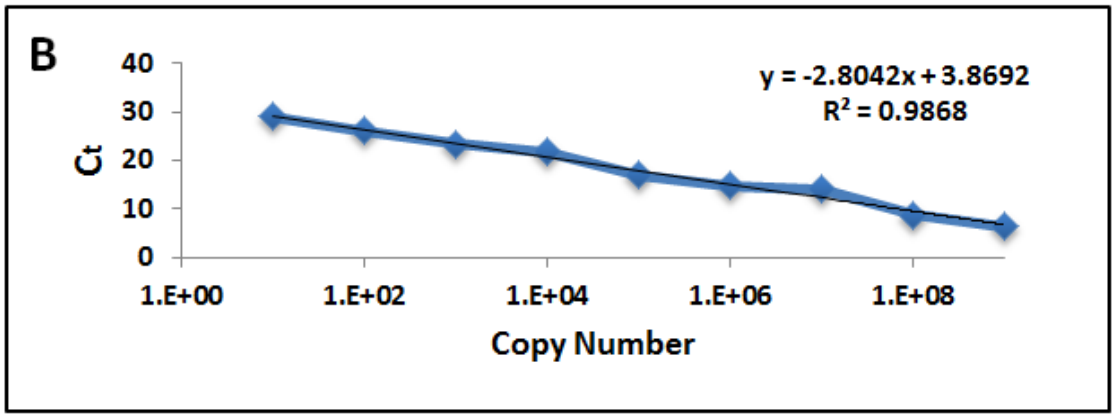


Figure 5.
(Left) Firefly luciferase activity statistically analyzed. Effects of wild-type RHA and RHA mutants K417R and W339A overexpression on Tax driven luciferase reporter plasmid expression in HEK293 cells. pCDNA (1 µg), wild-type RHA (1 µg) and RHA mutant (2 µg each) transfections were carried out first, followed by Tax (0.2 µg) plasmid transfection and HTLV-1 LTR-Luc (0.1 µg) plasmid cotransfection. The values obtained are averages of triplicates from 1 experiment. Error bars indicate standard deviation. (Right) The western blot analysis of exogenous RHA protein levels in cytoplasmic lysates of cells assuring equal exogenous RHA-protein expression.





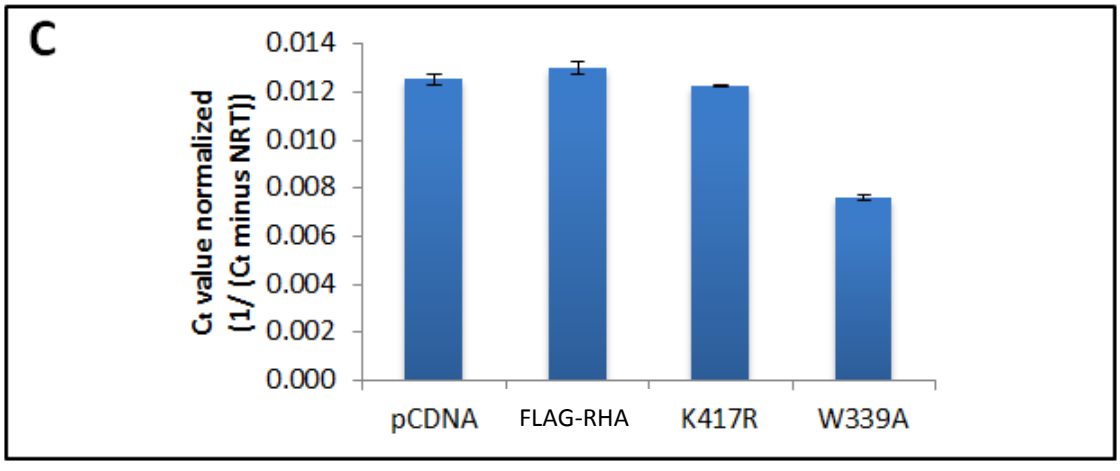
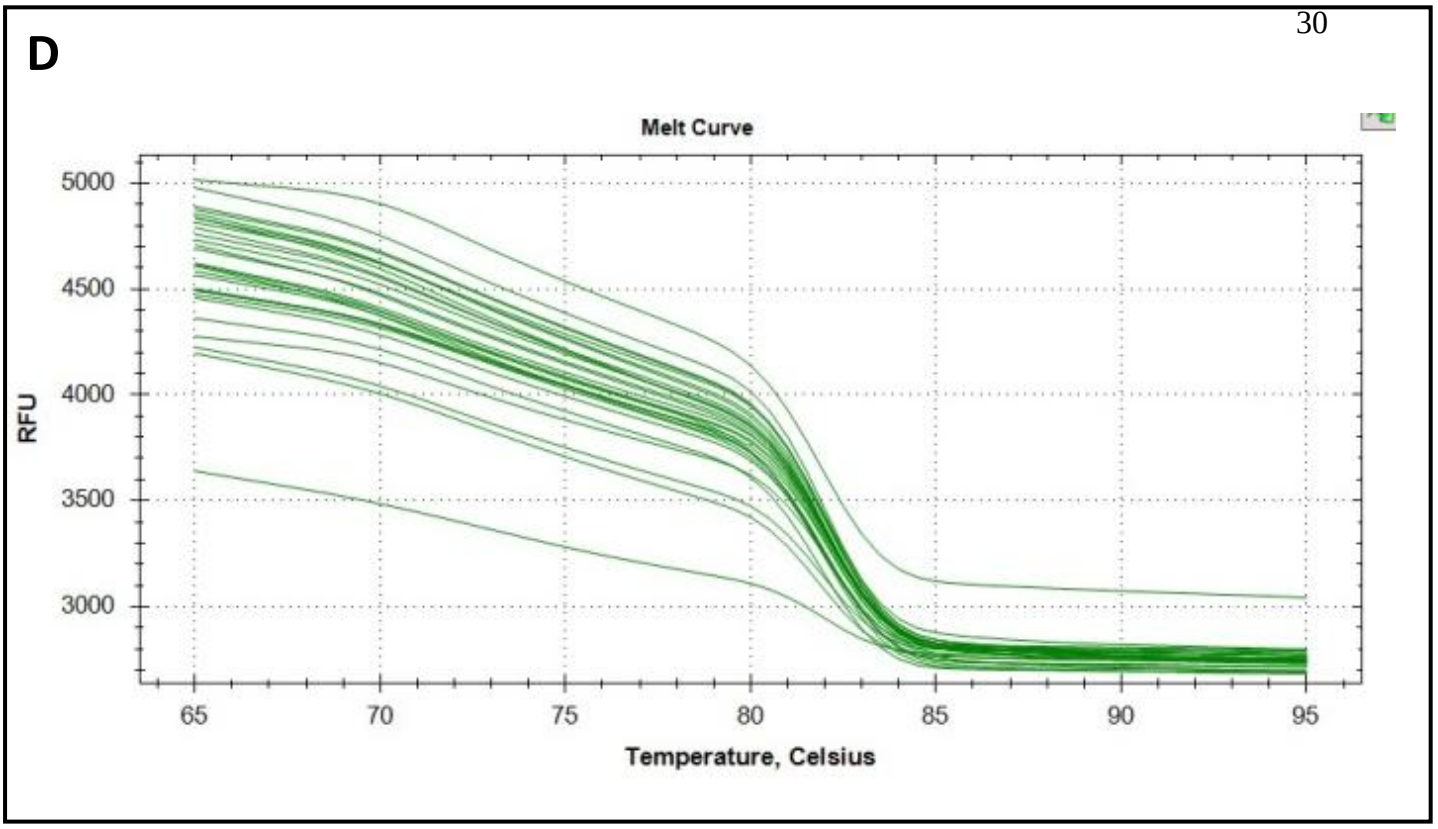


Figure 6.
(A) RNA analysis by RT-qPCR showed that steady state Luciferase mRNA levels were approximately the same with and without wild-type expression. RNA levels also decreased significantly when the mutants were overexpressed. **(B)** Standard curve for RT-PCR. **(C)** Threshold cycle (Ct) values of experimental samples normalized to samples with no reverse transcriptase (NRT).



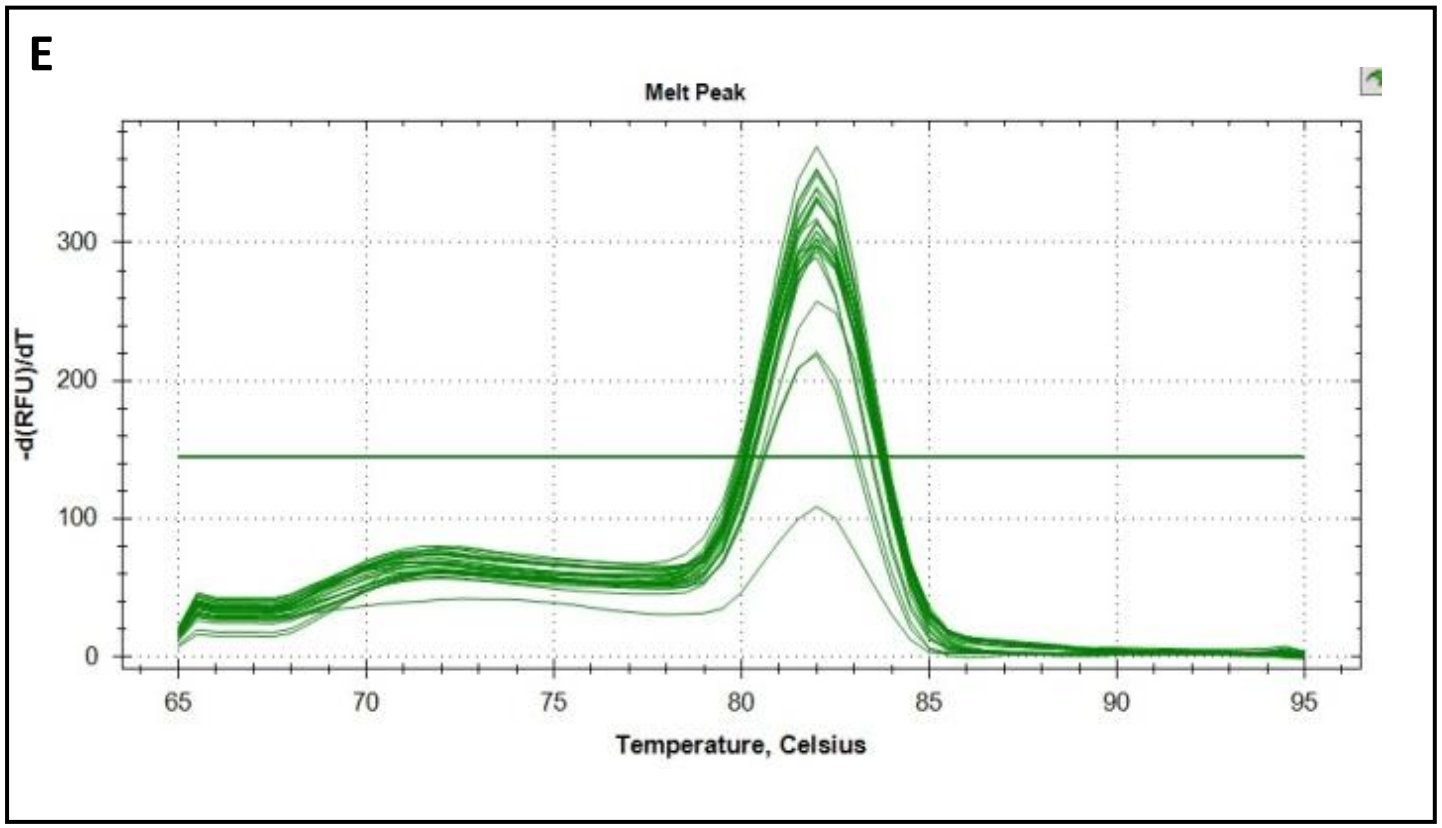


Figure 6 cont'd.
(D&E) Melting curves of PCR product to validate PCR accuracy and a lack of non-specific reaction products.

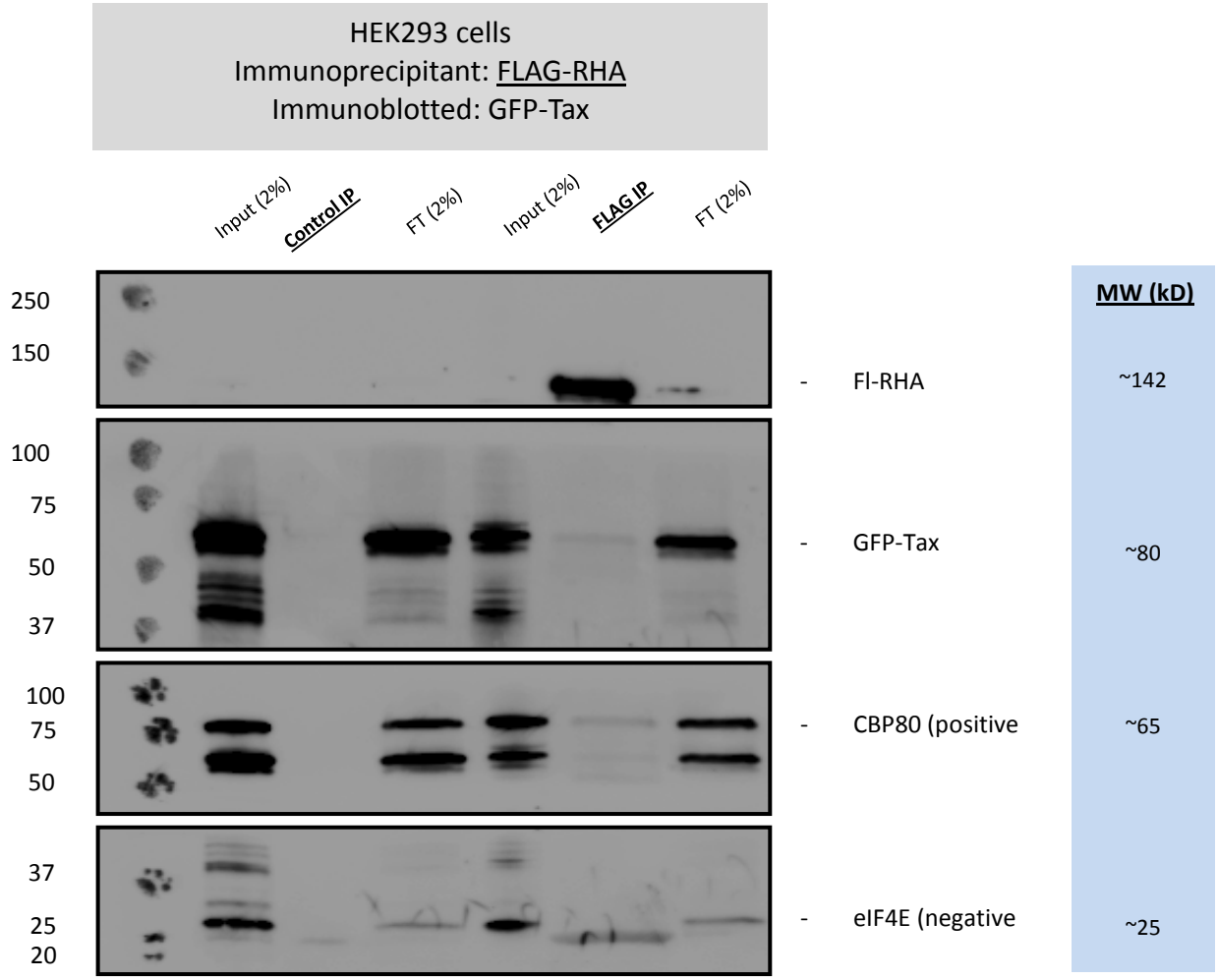


Figure 7.
RHA and Tax, as two proteins, have some affinity for and are capable of binding to each other in a biological setting. In our hands two controls that have been shown to bind or not bind to RHA – CBP80 (positive) and eIF4E (negative) – are used to validate a successful IP.

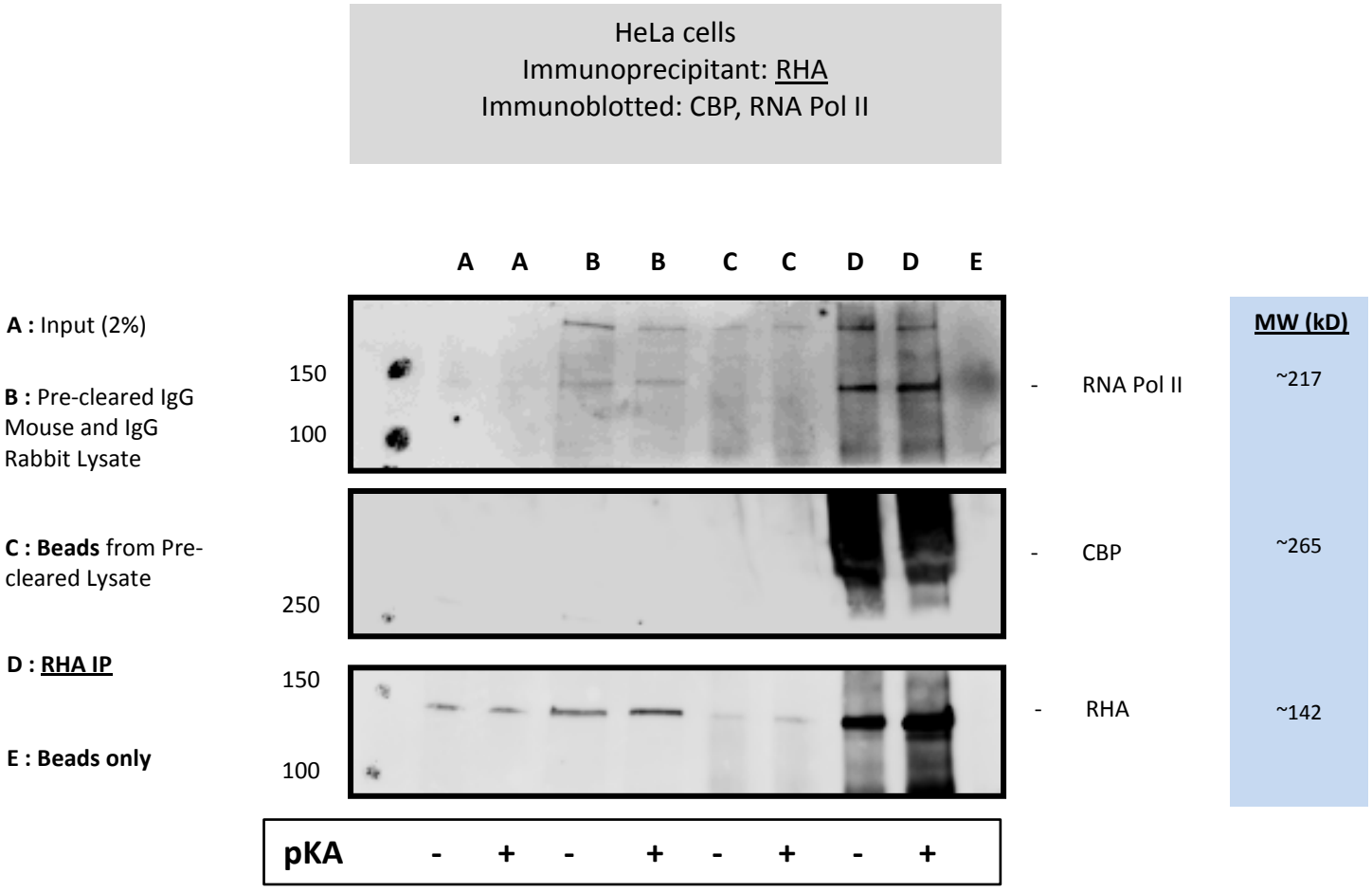


Figure 8.
Endogenous RHA forms a complex with endogenous CBP and endogenous RNA Polymerase II as supported by previous literature.

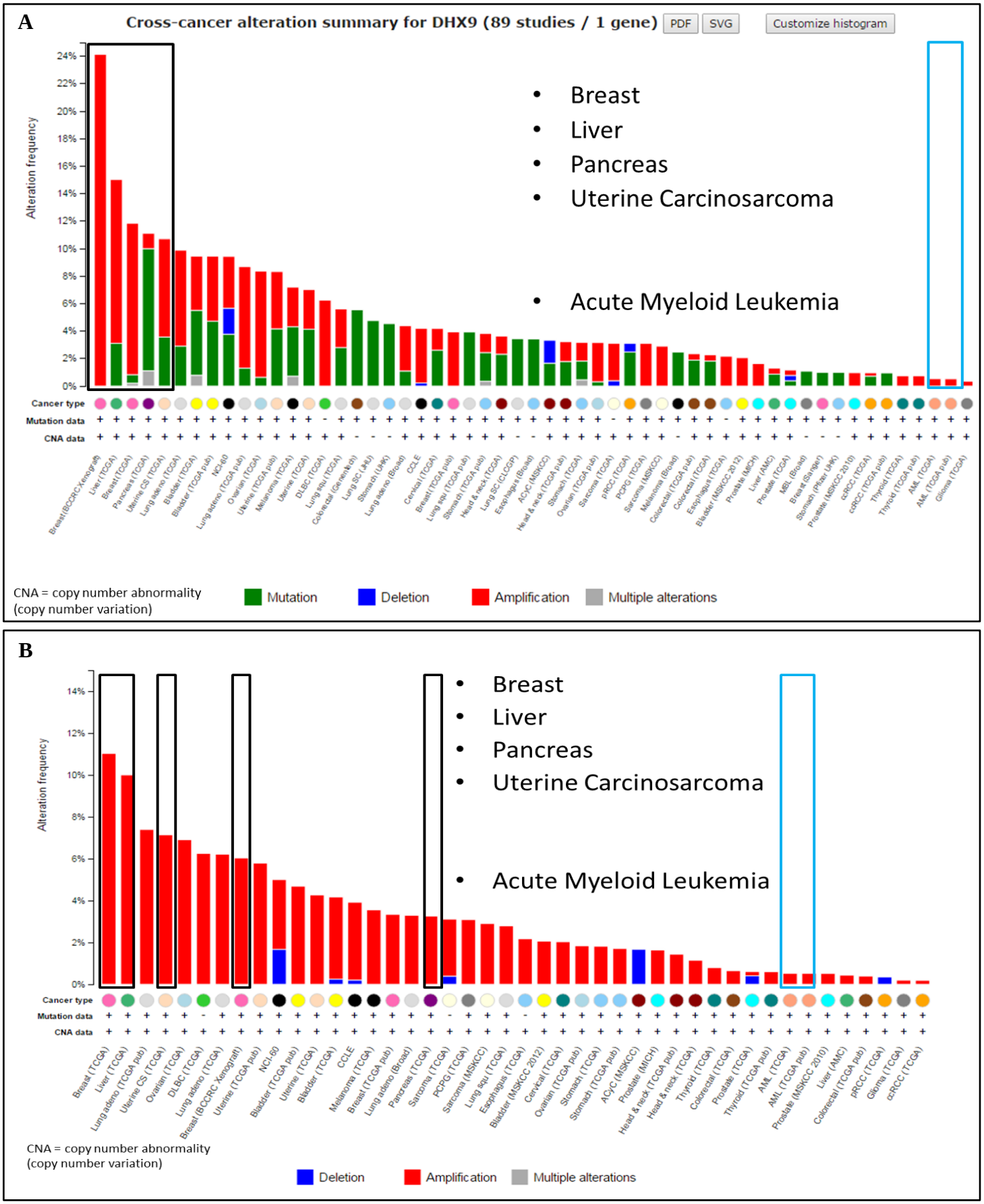
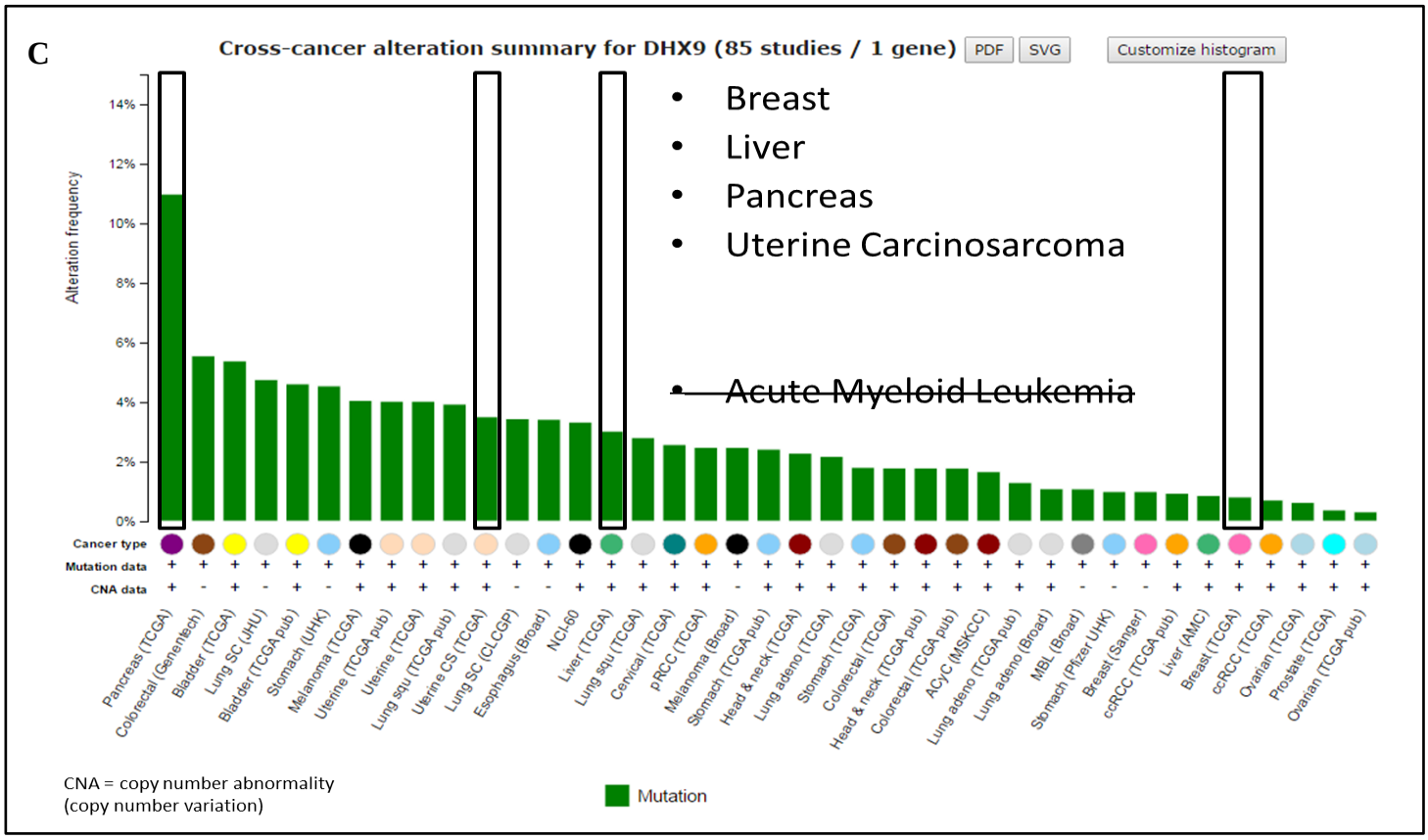
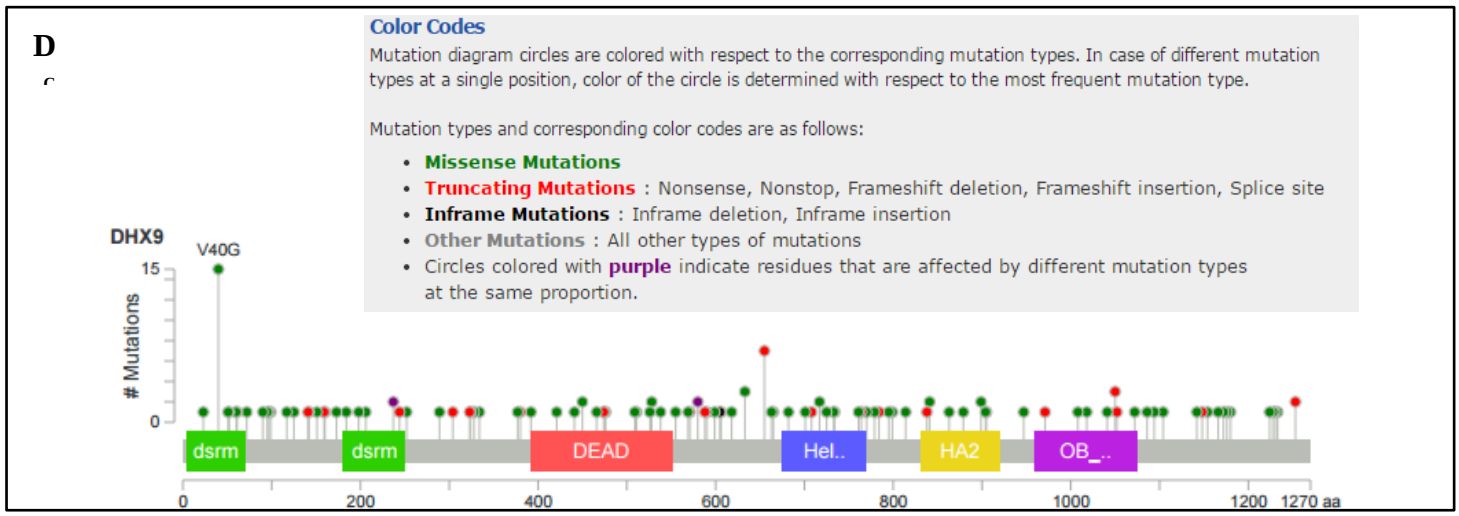


Figure 9.
(A) Five specific studies were selected based on having the highest "total alteration" frequencies.
(B) The same five studies among all cancer studies based on highest amplification or deletion type alteration frequencies.





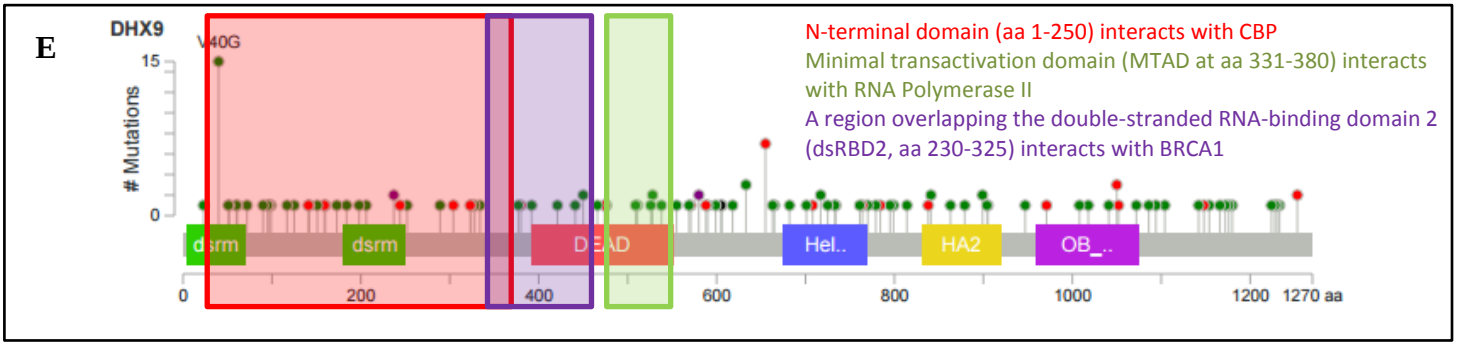


Figure 9 cont'd.
(C) The same five studies among all cancer studies based on highest mutation type alteration frequencies. **(D)**A plot of all known RHA mutations among cancer studies across the protein structure with a figure legend provided by the site. **(E)**Shaded boxes represent specific domains of RHA relevant to our study.

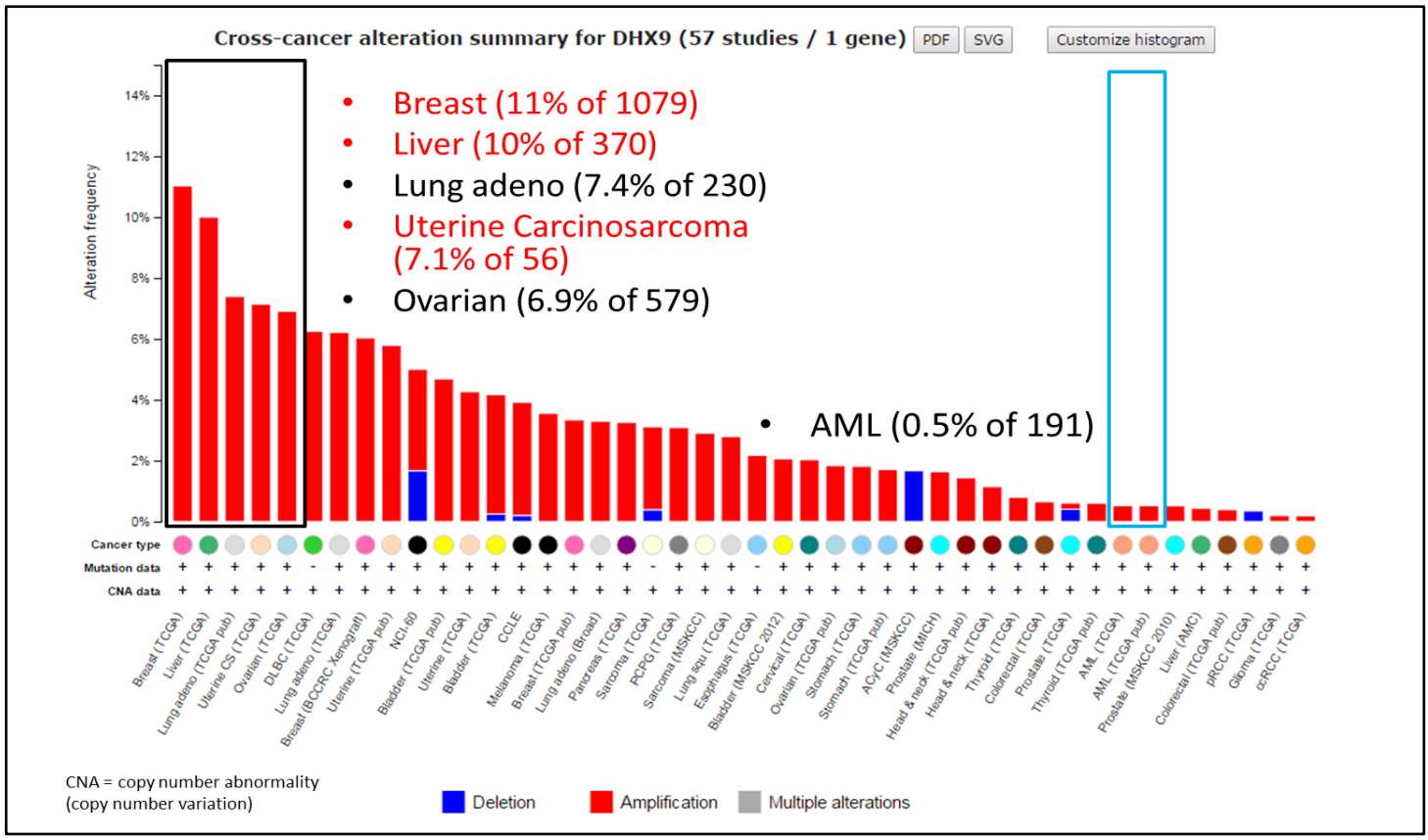


Figure 10.
Five studies among all cancer studies based on highest amplification or deletion type alteration frequencies. (Highlighted in red are cancers from among our previously selected five.)

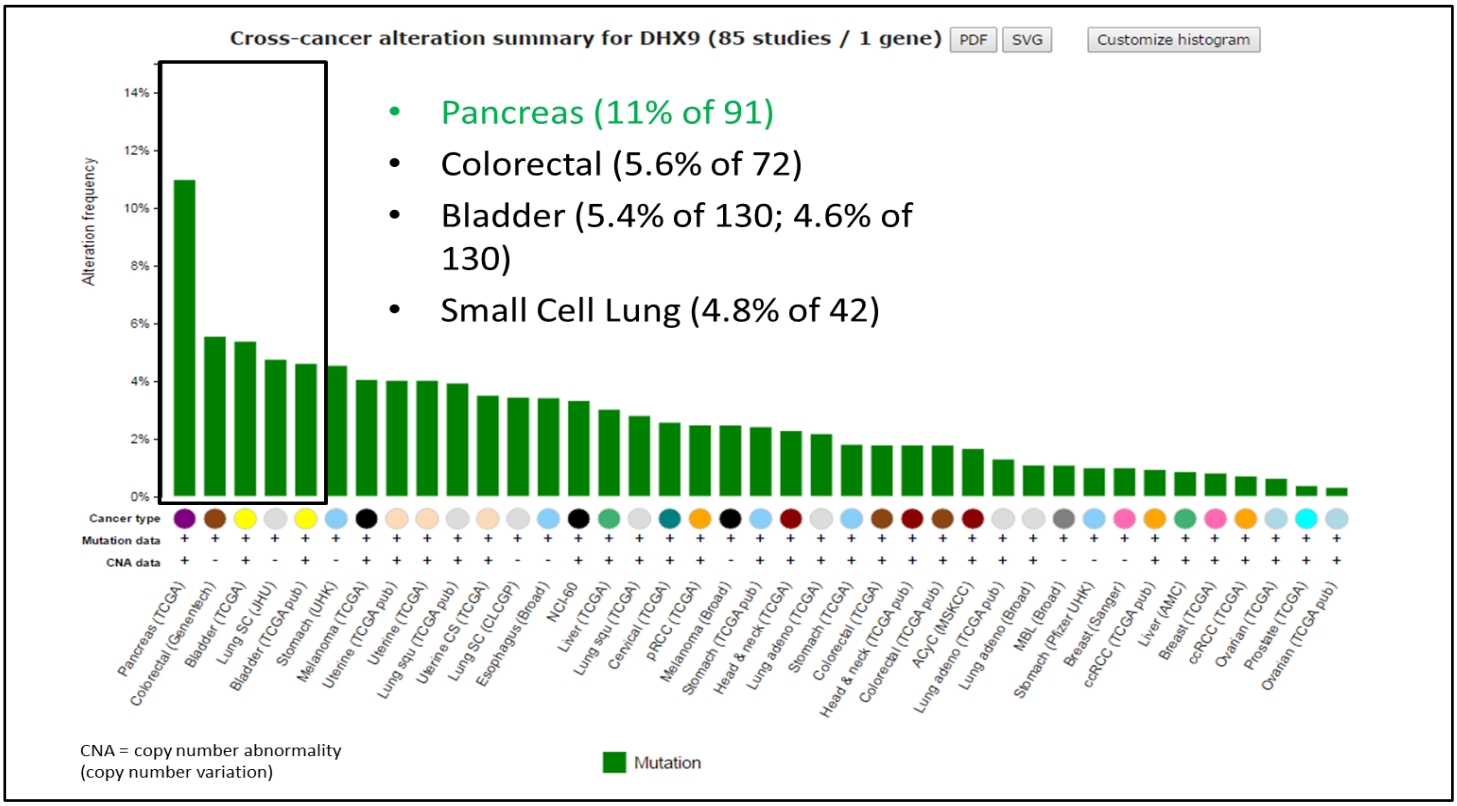
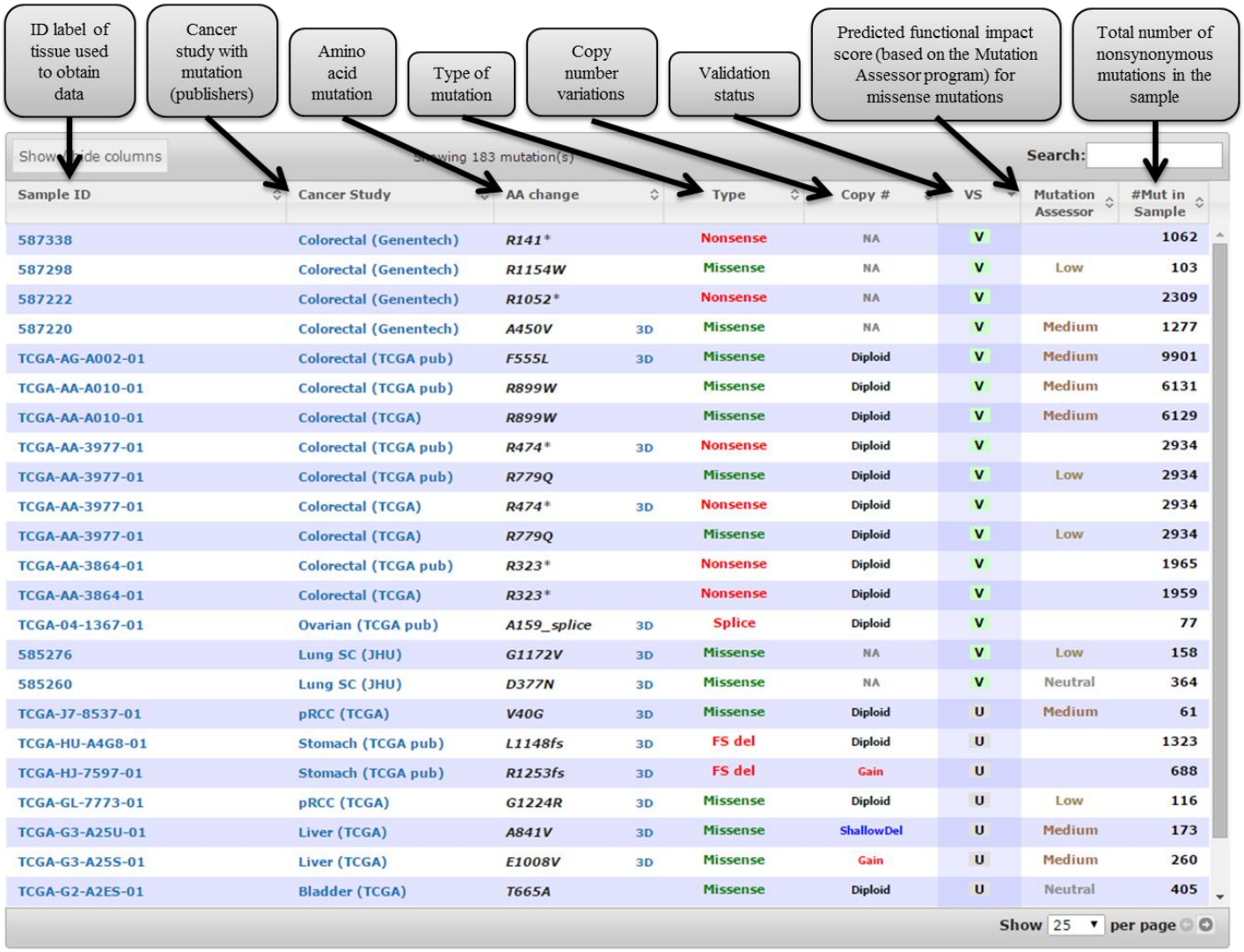


Figure 11.
Five studies among all cancer studies based on highest mutation type alteration frequencies. (Highlighted in green is a cancer from among our previously selected five.)



Sample ID	Cancer Study	AA change		Type	Copy #	VS	Mutation Assessor	#Mut in Sample
587338	Colorectal (Genentech)	*R141**		Nonsense	NA	V		1062
587298	Colorectal (Genentech)	*R1154W*		Missense	NA	V	Low	103
587222	Colorectal (Genentech)	*R1052**		Nonsense	NA	V		2309
587220	Colorectal (Genentech)	*A450V*	3D	Missense	NA	V	Medium	1277
TCGA-AG-A002-01	Colorectal (TCGA pub)	*F555L*	3D	Missense	Diploid	V	Medium	9901
TCGA-AA-A010-01	Colorectal (TCGA pub)	*R899W*		Missense	Diploid	V	Medium	6131
TCGA-AA-A010-01	Colorectal (TCGA)	*R899W*		Missense	Diploid	V	Medium	6129
TCGA-AA-3977-01	Colorectal (TCGA pub)	*R474**	3D	Nonsense	Diploid	V		2934
TCGA-AA-3977-01	Colorectal (TCGA pub)	*R779Q*		Missense	Diploid	V	Low	2934
TCGA-AA-3977-01	Colorectal (TCGA)	*R474**	3D	Nonsense	Diploid	V		2934
TCGA-AA-3977-01	Colorectal (TCGA)	*R779Q*		Missense	Diploid	V	Low	2934
TCGA-AA-3864-01	Colorectal (TCGA pub)	*R323**		Nonsense	Diploid	V		1965
TCGA-AA-3864-01	Colorectal (TCGA)	*R323**		Nonsense	Diploid	V		1959
TCGA-04-1367-01	Ovarian (TCGA pub)	*A159_splice*	3D	Splice	Diploid	V		77
585276	Lung SC (JHU)	*G1172V*	3D	Missense	NA	V	Low	158
585260	Lung SC (JHU)	*D377N*	3D	Missense	NA	V	Neutral	364
TCGA-J7-8537-01	pRCC (TCGA)	*V40G*	3D	Missense	Diploid	U	Medium	61
TCGA-HU-A4G8-01	Stomach (TCGA pub)	*L1148fs*	3D	FS del	Diploid	U		1323
TCGA-HJ-7597-01	Stomach (TCGA pub)	*R1253fs*	3D	FS del	Gain	U		688
TCGA-GL-7773-01	pRCC (TCGA)	*G1224R*	3D	Missense	Diploid	U	Low	116
TCGA-G3-A25U-01	Liver (TCGA)	*A841V*	3D	Missense	ShallowDel	U	Medium	173
TCGA-G3-A25S-01	Liver (TCGA)	*E1008V*	3D	Missense	Gain	U	Medium	260
TCGA-G2-A2ES-01	Bladder (TCGA)	*T665A*		Missense	Diploid	U	Neutral	405

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Figure 12.
cBioPortal's gene data can be organized into multiple categories. The current view is organized based on all "valid" mutations, in alphabetical and numerical orders depending on the cancer type first and then the sample ID.

	Overexpression of RHA increases HTLV-1 promoter activity					
HTLV-1 LTR-Luc	RNA (copy number/uL)	Significance (compared to pCDNA)	Protein (relative light units/uL)	Significance (compared to pCDNA)	Protein/RNA	Significance (compared to pCDNA)
pCDNA	676 ± 59	N/A	7188 ± 315	N/A	11 ± 1	N/A
FLAG-RHA	2961 ± 268	Significant ($p \leq 0.05$)	38976 ± 3184	Significant ($p \leq 0.05$)	13± 2	Not Significant ($p \geq 0.05$)
K417R	417 ± 5	Significant ($p \leq 0.05$)	4877 ± 1188	Significant ($p \leq 0.05$)	12 ± 3	Not Significant ($p \geq 0.05$)
W339A	157± 11	Significant ($p \leq 0.05$)	2546 ± 555	Significant ($p \leq 0.05$)	16 ± 3	Significant ($p \leq 0.05$)

Table 1.
The effects of RHA overexpression on luciferase RNA and protein expression.

Distribution of RHA alterations in tumor samples from TCGA						
	Any RHA alterations (percentage of total samples)	Total number with CNA and sequencing data	RHA CNA only data (percentage of total samples)	Total number with CNA data	* RHA tumor sequencing data (percentage of total samples)	* Total number with tumor sequencing data
Breast cancer patient xenografts	7 (24%)	29	7 (6%)	116	No Data	No Data
Acute Myeloid Leukemia	1 (0.5%)	191	1 (0.5%)	191	No Data	No Data
Liver hepatocellular carcinoma (TCGA unpublished)	29 (15%)	193	37 (10%)	370	6 (3%)	198
Breast invasive carcinoma (TCGA unpublished)	114 (12%)	962	119 (11%)	1079	8 (0.8%)	981
Pancreatic adenocarcinoma (TCGA unpublished)	10 (11%)	90	6 (3%)	184	10 (11%)	91
Uterine carcinosarcoma (TCGA unpublished)	6 (11%)	56	4 (7%)	56	2 (4%)	57
Acute Myeloid Leukemia (TCGA unpublished)	1 (0.5%)	188	1(0.5%)	191	No Data	No Data

Table 2.
Summary of data samples with RHA (DHX9) copy number alterations (CNA) and/or mutations among the selected five cancers and AML. This data is based off Figures 9A, 9B & 9C. * Tumor sequencing data is indicative of samples containing mutations.

Note: The cBioPortal site only stores mutation data for published cancer studies. Mutation data for provisional cancer data sets generate by The Cancer Genome Atlas (TCGA) are not stored. Provisional data is indicated above as TCGA unpublished; otherwise all data is published. National Cancer Institute guidelines prevent provisional data from being redistributed until they have been validated.

Mutations in selected domains				RHA alterations	
RHA domains and interacting partner	N-terminal domain (aa 1-250) interacting with CBP	MTAD domain (aa 331-380) interacting with RNA Pol II	dsRBD2 overlapping region (aa 230-325) interacting with BRCA1	Any RHA alterations (percentage of total samples)	Total number with CNA and sequencing data
Breast cancer patient xenografts	None	None	None	7 (24%)	29
Acute Myeloid Leukemia	None	None	None	1 (0.5%)	191
Liver hepatocellular carcinoma (Provisional)	D206G (medium)	None	None	29 (15%)	193
Breast invasive carcinoma (Provisional)	I23V (neutral)	None	None	114 (12%)	962
Pancreatic adenocarcinoma (provisional)	V40G (medium)	None	None	10 (11%)	90
Uterine carcinosarcoma (provisional)	L117R (medium)	None	None	6 (11%)	56
Acute Myeloid Leukemia (provisional)	None	None	None	1 (0.5%)	188

Table 3.
Commonly found mutations in specific domains of RHA in the selected five cancers and AML with their mutation assessor scores (netural, low, medium or high). This data is based off 9E and color coded according to 9D. The percentage of total RHA alterations from Table 2 is also shown.

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